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Biostable agonists that match or exceed activity of native insect kinins on recombinant arthropod GPCRs

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ABSTRACT

The multifunctional arthropod 'insect kinins' share the evolutionarily conserved C-terminal pentapeptide motif Phe-X^1-X^2-Trp-Gly-NH_2, where X^1 = His, Asn, Ser, or Tyr and X^2 = Ser, Pro, or Ala. Insect kinins regulate diuresis in many species of insects. Compounds with similar biological activity could be exploited for the control of arthropod pest populations such as the mosquito *Aedes aegypti* (L.) and the southern cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), vectors of human and animal pathogens, respectively. Insect kinins, however, are susceptible to fast enzymatic degradation by endogenous peptidases that severely limit their use as tools for pest control or for endocrinological studies. To enhance resistance to peptidases, analogs of the insect kinins incorporating bulky α,α-disubstituted amino acids in positions adjacent to both primary and secondary peptidase hydrolysis sites were synthesized. In comparison with a control insect kinin, several of these analogs are highly stable to hydrolysis by degradative enzymes ANCE, neprilysin and Leucine aminopeptidase. Six analogs were evaluated by calcium bioluminescence assay on recombinant receptors from mosquito and tick. Four of these analogs either matched or exceeded the potency of the control kinin peptide agonist. One of these was about 5-fold more potent than the control agonist on the tick receptor. This analog was 8-fold more potent than the control agonist on the mosquito receptor, and twice more potent than the endogenous *Aedes* kinin-II. The analog also demonstrated potent activity in an *in vitro Aedes* Malpighian tubule fluid secretion assay. Similar comparisons of analog potency cannot be made to tick kinins because no endogenous kinin has yet been identified. These potent, biostable analogs represent ideal new tools for endocrinologists studying arthropod kinin-regulated processes *in vivo*, particularly for ticks in which their role remains to be established.

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1. Introduction

G-Protein-coupled receptors (GPCRs) are seven transmembrane cell surface proteins that are activated by diverse stimuli such as biogenic amines, neuropeptides and protein hormones. A distinct intracellular response, mostly through their heterotrimeric G-protein, is initiated when GPCRs are activated by their ligands leading to biological effects (Park and Adams, 2005; Kristiansen, 2004; Pierce et al., 2002; Han et al., 2007). Insects have 50–80 neurohormone GPCRs, which, together with their ligands, control most critical metabolic processes, such as reproduction, development, homeostasis and feeding (Grimmelikhuijzen et al., 2007; Hauser et al., 2006a,b; Gäde and Goldsworthy, 2003; Gäde, 2004). Because these receptors are involved in critical physiological processes they are considered good targets to control arthropod pest population. Blocking or overstimulating these receptors could lead to reduction of pest fitness or death (Gäde and Goldsworthy, 2003).

Insect neuropeptides found in several arthropod and invertebrate groups regulate important biological functions including water balance, egg and pheromone production, blood sugar level, metamorphosis, etc. (Bede et al., 2007; Coast, 2007; Coast et al., 2002; De Loof, 2008; Gäde, 2004; Nässel, 1996; Riehle et al., 2002; Torfs et al. 1999; Altstein, 2004). In diverse species, insect kinins stimulate hindgut contractions, diuresis, digestive enzyme release and probably inhibit larval weight gain (Holman et al., 1990; Coast et al., 1990; Nachman et al, 1991, 2002; Harshini el al., 2002, 2003; Seinsche et al., 2000). Due to their specificity and their high activity at extremely low dosages, neuropeptides have been studied as potential leads for the development of new

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14. ABSTRACT

The multifunctional arthropod ?insect kinins? share the evolutionarily conserved C-terminal pentapeptide motif Phe-X1-X2-Trp-Gly-NH2, where X1 = His, Asn, Ser, or Tyr and X2 = Ser, Pro, or Ala. Insect kinins regulate diuresis in many species of insects. Compounds with similar biological activity could be exploited for the control of arthropod pest populations such as the mosquito Aedes aegypti (L.) and the southern cattle tick Rhipicephalus (Boophilus) microplus (Canestrini), vectors of human and animal pathogens respectively. Insect kinins, however, are susceptible to fast enzymatic degradation by endogenous peptidases that severely limit their use as tools for pest control or for endocrinological studies. To enhance resistance to peptidases, analogs of the insect kinins incorporating bulky a,a-disubstituted amino acids in positions adjacent to both primary and secondary peptidase hydrolysis sites were synthesized. In comparison with a control insect kinin, several of these analogs are highly stable to hydrolysis by degradative enzymes ANCE, neprilysin and Leucine aminopeptidase. Six analogs were evaluated by calcium bioluminescence assay on recombinant receptors from mosquito and tick. Four of these analogs either matched or exceeded the potency of the control kinin peptide agonist. One of these was about 5-fold more potent than the control agonist on the tick receptor. This analog was 8-fold more potent than the control agonist on the mosquito receptor, and twice more potent than the endogenous Aedes kinin-II. The analog also demonstrated potent activity in an in vitro Aedes Malpighian tubule fluid secretion assay. Similar comparisons of analog potency cannot be made to tick kinins because no endogenous kinin has yet been identified. These potent, biostable analogs represent ideal new tools for endocrinologists studying arthropod kinin-regulated processes in vivo, particularly for ticks in which their role remains to be established.

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environmentally friendly pest control agents. However, the natural compounds cannot be directly used, as they are susceptible to degradation by endogenous peptidases present in the insect digestive system and circulating hemolymph (blood) (Cornell et al., 1995; Gäde and Goldsworthy 2003; Lamango et al., 1996). If both chemical and conformational requirements responsible for neuropeptide biological activity were fully understood, design of analogs containing unnatural moieties could overcome these limitations (Nachman et al., 1994).

The endogenous arthropod insect kinins are 6–14 amino acids long neuropeptides characterized by the evolutionarily conserved C-terminal pentapeptide Phe-X^1-X^2-Trp-Gly-NH_2, where X^1 = His, Asn, Ser, or Tyr and X^2 = Ser, Pro, or Ala (Holman et al., 1999; Torfs et al., 1999). This C-terminal pentapeptide kinin core is the minimum sequence required for full cockroach myotropic and cricket diuretic activity in *in vitro* tissue assays (Nachman and Holman, 1991; Nachman et al., 2003) and for bioluminescence response in CHO-K1 cells expressing kinin receptors (Holmes et al., 2003; Pietrantonio et al., 2005; Taneja-Bageshwar et al., 2006). Both the tissue assays and the receptor expressing system revealed that the C-terminal amide of the insect kinin is important for their activity. Activity of the insect kinin core was completely lost when the C-terminal amide was replaced with the negatively charged acid moiety (Nachman et al., 1995; Taneja-Bageshwar et al., 2006). Activity was also completely lost in these assay systems when either Phe^1 or Trp^4 was replaced with Ala, confirming the importance of these two key positions (Taneja-Bageshwar et al., 2006). However, the variable position 2 tolerates a wide range of chemical characteristics, from acidic to basic residues, and from hydrophilic to hydrophobic, although highest potencies in Malpighian tubule fluid secretion assays and receptor expressing systems were observed with aromatic residues at this position (Nachman and Holman, 1991; Roberts et al., 1997; Taneja-Bageshwar et al., 2006). Based on these observations the plausible receptor interaction model positions the side chains of Phe^1 and Trp^4 towards the same region where they interact with the receptor, and away from the side chain of position 2.

In vivo, kinins are subjected to rapid biological degradation. Experimentally, the angiotensin-converting enzyme (ACE) from the housefly can cleave the insect kinin primary hydrolysis site and neprilysin (NEP) can cleave both the primary and secondary hydrolysis sites (Cornell et al., 1995; Lamango et al., 1996; Nachman et al., 2002, 1997a,b, 1990; Roberts et al., 1997). Therefore, pseudopeptides with enhanced resistance to peptidases that retain biological activity on 'insect kinin' receptors of arthropod vectors at a potency that matches native or control peptide agonists are needed. Towards this purpose, incorporation of a single α-amino isobutyric acid (Aib) into the third position of the insect kinin active core effectively protects the primary hydrolysis site from tissue-bound peptidase (Nachman et al., 1997a,b, 2002; Taneja-Bageshwar et al., 2006). Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site further enhances biostability (Nachman et al., 2002).

The availability of kinin receptors from the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Holmes et al., 2000, 2003), and the dengue vector, the mosquito *Aedes aegypti* (Pietrantonio et al., 2005), stably expressed in CHO-K1 cells, allows the opportunity to evaluate a new series of kinin analogs featuring enhanced biostability. These analogs that incorporate sterically bulky α,α-disubstituted amino acid residues, such as α-amino isobutyric acid (Aib), adjacent to both primary and secondary peptidase hydrolysis sites were evaluated through a functional calcium bioluminescence assay on the two recombinant receptors. Six such analogs were synthesized in which the third position (S or P), adjacent to the primary peptidase hydrolysis site, was replaced with Aib. In these analogs, a position adjacent to the secondary peptidase hydrolysis site was also replaced with either an α,α-disubstituted amino acid or a β-amino acid that leads to an enhancement of biostability (see Fig. 1). These analogs are:

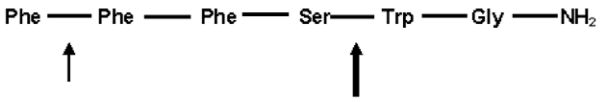

Fig. 1. Primary (large arrow) and secondary (small arrow) hydrolysis sites of tissue-bound peptidase for the insect kinin FFFSWGa, adapted from Lamango et al., 1997.

K-Aib-1 [Aib]FF[Aib]WGa
K-Aib-2 [α MeF]FF[Aib]WGa
K-Aib-3 Ac-R[Aib]FF[Aib]WGa
K-Aib-4 Ac-R[β3F]FF[Aib]WGa
K-Aib-5 [Aib]RFF[Aib]WGa
K-Aib-6 [Aib-Aib-Aib-Aib]RFF[Aib]WGa
where K stands for Kinin analog.

2. Materials and methods

2.1. Analog synthesis and purification

Analogs were synthesized on an ABI 433A peptide synthesizer with a modified FastMoc 0.25 procedure using an Fmoc-strategy starting from Rink amide resin (Novabiochem, San Diego, CA, 0.5 mM/g). The Fmoc protecting group was removed by 20% 4-methyl piperidine in DMF (Dimethyl formamide). A 4-fold excess of the respective Fmoc-amino acids was activated *in situ* using HBTU (2-(1h-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (1 eq.)/HOBt (1-hydroxybenzotriazole) (1 eq.) in NMP (*N*-methylpyrrolidone) or HATU (2-(7-Aza-1H-Benzotiazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (1 eq.)/HOAt (1-hydroxy-7-azabenzotriazole) (1 eq.) in NMP for Aib and the amino acid immediately following it in the sequence. The coupling reactions were base catalyzed with DIPEA (*N*,*N*-diisopropylethylamine) (4 eq.) The amino acid side-chain protecting groups were PMC for Arginine and Boc for Tryptophan. The analogs were cleaved from the resin with side-chain deprotection by treatment with TFA (Trifluoroacetic acid):H_2O:TIS (Triisopropylsilane) (95.5:2.5:2.5 v/v/v) for 1.5 h. The solvents were evaporated by vacuum centrifugation and the analogs were desalted on a Waters C_{18} Sep Pak cartridge (Milford, MA) in preparation for purification by HPLC.

The analogs were purified on a Waters Delta-Pak C_{18} reverse-phase column (8 × 100 mm, 15 μm particle size, 100 Å pore size) with a Waters 510 HPLC system with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous TFA; Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Initial conditions were 10% B followed by a linear increase to 90% B over 40 min.; flow rate, 2 ml/min. Delta-Pak C_{18} retention times: **K-Aib-1**, [Aib]F-F[Aib]WGa, 15.0 min.; **K-Aib-2**, [αMeF]FF[Aib]WGa, 16.0 min.; **K-Aib-3**, Ac-R[Aib]FF[Aib]WGa, 16.4 min.; **K-Aib-4**, Ac-R[β^3F]FF[Aib]WGa, 17.0 min.; **K-Aib-5**, [Aib]RFF[Aib]WGa, 14.0 min.; **K-Aib-6**, [Aib-Aib-Aib-Aib]RFF[Aib]WGa, 16.4 min. The analogs were further purified on a Waters Protein Pak I 125 column (7.8 × 300 mm). Conditions: isocratic using 80% acetonitrile containing 0.1% TFA; flow rate, 2 ml/min. Waters Protein Pak retention times: **K-Aib-1**, 5.0 min.; **K-Aib-2**, 5.9 min.; **K-Aib-3**, 4.5 min.; **K-Aib-4**, 6.0 min.; **K-Aib-5**, 6.0 min.; **K-Aib-6**, 6.0 min. Amino acid analysis was carried out under previously reported conditions (Nachman et al., 2004) to quantify the analogs and to confirm identity: **K-Aib-1**: F[1.0], F[1.0], G[0.7]; **K-Aib-2**: F[1.0], F[1.0], G[0.9]; **K-Aib-3**: R[0.9], F[1.0], F[1.0], G[0.8]; **K-Aib-4**: R[0.9], F[1.0], F[1.0], G[0.9];

K-Aib-5: R[1.2], F[1.0], F[1.0], G[0.9]; **K-Aib-6**: R[1.1], F[1.0], F[1.0], G[1.0]. The identity of the analogs was also confirmed by MALDI-MS on a Kratos Kompact Probe MALDI-MS instrument (Shimadzu, Columbia, Maryland). The following molecular ions (MH^+) were observed: **K-Aib-1**, 726.8 (calc. 724.2); **K-Aib-2**, 802.3 (800.21 calc.); **K-Aib-3**, 924.1 (calc. 923.3); **K-Aib-4**, 1000.1 (calc. 999.2); **K-Aib-5**, 882.6 (calc. 881.3); **K-Aib-6**, 1137 (calc. 1136.3).

2.2. Cell lines

Receptor cloning, transfection and selection of single clonal cell lines expressing the kinin receptors from the southern cattle tick, *B. microplus* (AF228521) (leucokinin-like receptor) and the yellow fever mosquito, *A. aegypti* (AY596453) was reported previously (Holmes et al., 2000, 2003; Pietrantonio et al., 2005). The CHO-K1 cell lines expressing, respectively, the tick receptor, BmLK3 (Holmes et al., 2003), and the *Aedes* kinin receptor, E10 (Pietrantonio et al., 2005), were maintained in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum (EquiTech Bio, Kerrville, TX) with 400 mg/ml GENETICIN at 37 °C and 5% CO_2.

2.3. Analysis of activity of Aib-containing analogs of insect kinin peptide by a Ca^{2+} bioluminescence plate assay

The functional analysis of Aib-containing insect kinin analogs with stably transformed CHO-K1 cells expressing the tick or mosquito receptor was performed by an intracellular calcium bioluminescence assay as described previously (Pietrantonio et al., 2005; Taneja-Bageshwar et al., 2006). This assay uses a photoprotein isolated from jelly fish (*Aequorea victoria*), composed of an apoprotein (apoaequorin) and a prosthetic group coelenterazine; holoprotein aequorin is formed in the presence of oxygen. When aequorin comes in contact with calcium, it undergoes a conformational change, filling up the calcium binding sites on the protein. This releases oxygen that in turn oxidizes coelenterazine to excited coelenteramide. When excited coelenteramide relaxes to the ground state it emits light at 469 nm (Mithofer and Mazars, 2002). Transient transfection with aequorin was as described previously (Taneja-Bageshwar et al., 2006, 2008a,b). The CHO-K1 cells expressing the kinin receptors were grown in F12K media containing 10% fetal bovine serum and 400 mg/ml GENETICIN® to about 90% confluency in T-25 flasks at 37 °C and 5% CO_2. Cells were trypsinized and seeded in each well of 6-well tissue culture plate at a density of 2×10^5 cells in 2 ml of media. For a typical assay, 2–3 wells were sufficient. Cells were allowed to grow to about 60% confluency for 24 h at 37 °C and 5% CO_2. Next day, media was replaced with reduced serum OPTI-MEM media (Gibco, Invitrogen Co.). For transfection of cells with aequorin, 96 μl of OPTI-MEM media was mixed with 4 μl of the transfection reagent Fugene 6 (Roche Biochemicals) in a microfuge tube for each well of 6-well plate. This mixture was incubated for 5 min at room temperature. To this mix 1 μg of aequorin/pcDNA1 plasmid DNA in 10 mM Tris buffer (pH 8.5), without EDTA was added and incubated for another 15 min at room temperature. After 15 min the mixture was added dropwise to each well with gentle manual shaking and the plates were incubated for 4–6 h at 37 °C and 5% CO_2. The media was changed to F12 K media containing 10% fetal bovine serum without antibiotic and cells were grown overnight at 37 °C and 5% CO_2. The next day, cells were trypsinized and transferred to 96-well, white, thin bottom micro titer plates (Costar 3610) at a density of 40,000 cells/100 μl per well for testing the analogs. To reconstitute the aequorin complex, on the day of assay, cells were incubated in (90 μl/well) calcium-free DMEM media (GIBCO, Invitrogen Co., CA) containing 5 μM coelenterazine (Molecular Probes, Invitrogen Co., CA) for 3 h in the dark at 37 °C and 5% CO_2 (Stables et al., 1997). Cells were then challenged with different concentrations of analogs in a volume of 10 μl (10×) solubilized in calcium-free DMEM media. In all the assays, FFFSWGa a potent hexapeptide was used as a positive control for both the cell lines (Taneja-Bageshwar et al., 2006). The assay was performed using the NOVOstar (BMG Labtechnologies) plate reader in bioluminescence mode at room temperature. Light emission (469 nm) was recorded every 2 s over a period of 50 s per well. Each analog was repeated at least three times with two replicates each. Concentration response curves were obtained by nonlinear regression curve fit analysis (sigmoidal dose–response equation with variable slope) using Prism software 4.0. Maximal responses from six individual replicates at each of 11 concentrations from 1 nM to 10 μM were used for calculations of the EC_{50}. Statistical analysis was performed by one-way analysis variance (ANOVA) with Tukey multiple Comparison Test (GraphPad, Prism version 4.0, San Diego, CA).

2.4. Enzyme degradation assays

The enzyme degradation assays were performed on those three analogs that were most potent on both the receptors. The assays were performed as reported previously (Zubrzak et al., 2007; Cornell et al., 1995; Lamango et al., 1996). In short, peptide analogs (10 μM) were incubated with either angiotensin-converting enzyme (ANCE) (0.5–2 ng), leucine aminopeptidase (Sigma–Aldrich, Type VI; 0.3–10 ng) or human neprilysin (10–100 ng) in 0.1 M Hepes buffer, pH 7.0 (total volume, 20 μl) at 35 °C. Reactions were terminated by the addition 5 μl of 8% TFA and in preparation for HPLC analysis the volume was increased to 260 μl by the addition of 0.1% TFA. Percent hydrolysis of each peptide was determined by using HPLC to measure the amount of the parent peak remaining after incubation. Results are the mean of three individual assays. HPLC was performed using a Jupiter 5μ, column (C18, 250 mm in length × 4.5 mm, internal diameter) and a linear solvent gradient of 24% rising to 50% of acetonitrile in 0.1% TFA over 20 min at flow rate of 1 ml/min.

2.5. Malpighian tubule fluid secretion assay

Malpighian tubules were removed from adult females (*Aedes aegypti*) at 3–10 days post-emergence. Tubule secretion assays were performed as described by Clark et al. (1998), but using 9 μl drops of bathing fluid with the following composition (in mmol/l): NaCl, 150; $NaHCO_3$, 1.8; KCl, 3.4; $CaCl_2$, 1.7; $MgSO_4$, 4.1; glucose, 5; HEPES (*N*-2-hydroxyethylpiperazine-*Ń*2-ethanesulphonic acid), 25; pH adjusted to 7.1 with 1 mol/l NaOH. The control rate of secretion was measured over 30 min after which test compounds were added to the bathing fluid dissolved in 1 μl of saline at 10-times the required concentration. Stimulated rates of secretion were then measured over two 15 min periods and the average rate of secretion (in μl/min) expressed as a percentage of the control rate.

3. Results

Effect of substitution of α-amino isobutyric acid on the activity of insect kinin C-terminal pentapeptide core by calcium bioluminescence plate assay

Aequorin-based functional calcium bioluminescence plate assay was used to study the effect of Aib substitution on the activity of insect kinin C-terminal pentapeptide core FFSWGa. Six Aib analogs of the pentapeptide core were synthesized and tested on stable tick (BmLK3 cell line) and mosquito insect kinin receptor (E10 cell line) expressing CHO-K1 cell lines. All the analogs were tested from 1 to 10 μM final concentration.

On both tick and mosquito receptors, all six analogs were active. On tick receptor, analog **K-Aib-1**, [Aib]FF[Aib]WGa was the most

active and **K-Aib-6**, [Aib-Aib-Aib-Aib]RFF[Aib]WGa was the least active analog. All the analogs were as efficacious as the positive control, FFFSWGa, in eliciting the response but varied in their potencies (Fig. 2 and Table 2). Determination of EC_{50} values revealed the order of potency as **K-Aib-1**, EC_{50} = 49 nM > **K-Aib-2**, [αMeF]FF[Aib]WGa, EC_{50} = 111 nM > **K-Aib-3**, Ac-R[Aib]FF[Aib]WGa, EC_{50} = 112 nM > **K-Aib-4**, Ac-R[β^3F]FF[Aib]WGa, EC_{50} = 211 nM > FFFSWGa, EC_{50} = 271 nM > **K-Aib-5**, [Aib]RFF[Aib]WGa, EC_{50} = 299 nM > **K-Aib-6**, EC_{50} = 624 nM (Fig. 2). Statistical analysis showed analog **K-Aib-1** to be statistically more potent than the control agonist FFFSWGa, and analogs **K-Aib-1**, **K-Aib-3** and **K-Aib-2** to be statistically more potent than analog **K-Aib-6**. The analog **K-Aib-1** is more than 5-fold more potent than the control, FFFSWGa.

On the mosquito receptor, the most and least active analogs were the same as for the tick receptor; analog **K-Aib-1** was the most potent and analog **K-Aib-6** was the least potent of all six analogs tested. All the analogs were as efficacious as the positive control, FFFSWGa, but varied in their potencies. Determination of EC_{50} values revealed the order of potency as **K-Aib-1**, EC_{50} = 76 nM > **K-Aib-2**, [αMeF]FF[Aib]WGa, EC_{50} = 289 nM > **K-Aib-4**, Ac-R[β^3F]FF [Aib]WGa, EC_{50} = 411 nM > FFFSWGa, EC_{50} = 617 nM > **K-Aib-3**, Ac-R[Aib]FF[Aib]WGa, EC_{50} = 625 nM = **K-Aib-5**, [Aib]RFF[Aib]WGa, EC_{50} = 625 nM > **K-Aib-6**, EC_{50} = 814nM (Fig. 3, Table 2). Statistical analysis showed analog **K-Aib-1** to be significantly different from analogs **K-Aib-3**, **K-Aib-4**, **K-Aib-5**, FFFSWGa, and **K-Aib-6;** and was also significantly different from *Aedes* kinin-II (data not shown). The order of activity for these analogs on the mosquito receptor deviated from that observed for the tick receptor only in that the order of potency for analogs **K-Aib-3** and **K-Aib-4** were switched. Analog **K-Aib-1** was found to be 8-fold more potent than control analog, FFFSWGa, and also more potent than the native *Aedes* kinin-II, EC_{50} = 164 nM (Table 2) (Taneja-Bageshwar et al., 2008b).

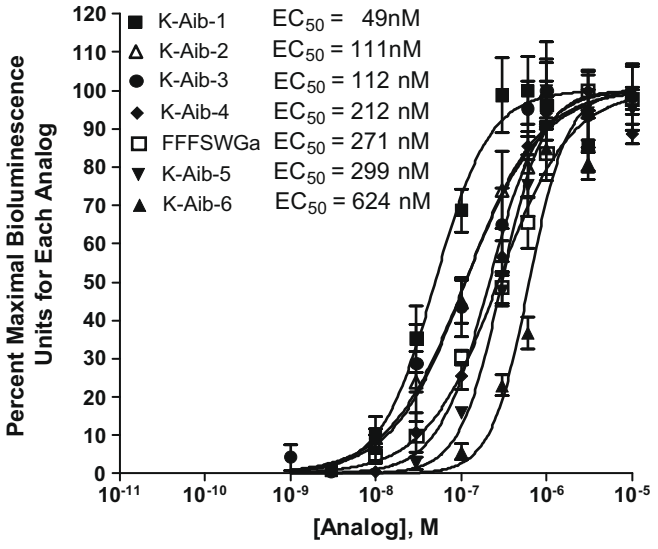

Fig. 2. Activity comparison of six α-amino isobutyric acid analogs on the tick kinin receptor expressing cell line by a calcium bioluminescence plate assay. The *y*-axis represents percent maximal bioluminescence units for each analog expressed as a percentage of bioluminescence observed at a concentration versus the maximal response observed among all concentrations tested for each analog. Statistical analysis and graphs were created with GraphPad Prism 4.0 software. Vertical lines on graph represent standard errors of independent experiments from maximum of six to minimum of three repetitions each consisting of two wells each. Analog FFFSWGa is the positive control for the receptor activity.

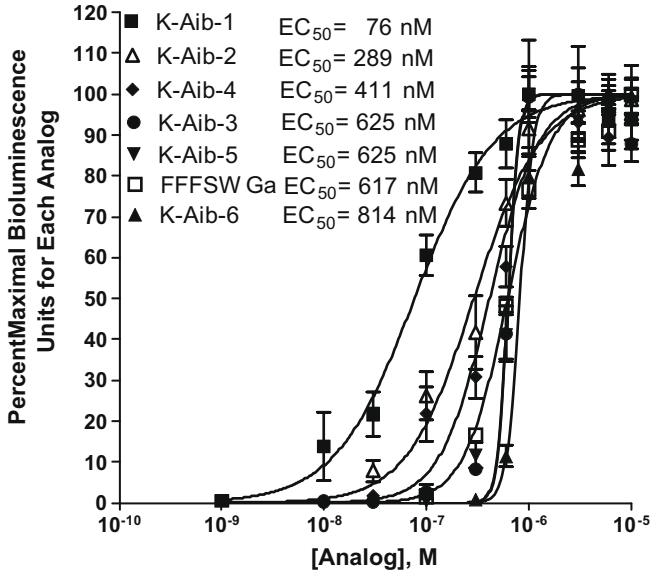

Fig. 3. Activity comparison of six α-amino isobutyric acid analogs on the mosquito kinin receptor expressing cell line by a calcium bioluminescence plate assay. The *y*-axis represents percent maximal bioluminescence units for each analog expressed as a percentage of bioluminescence observed at a concentration versus the maximal response observed among all concentrations tested for each analog. Statistical analysis and graphs were created with GraphPad Prism 4.0 software. Vertical lines on graph represent standard errors of independent experiments from maximum of six to minimum of three repetitions each consisting of two wells each. Analog FFFSWGa is the positive control for the receptor activity.

3.1. Enzyme degradation assays

A comparison of the rates of hydrolysis of a select group of the Aib-containing insect kinin analogs with the standard agonist FFFSWGa indicates that all are significantly more stable (see Table 1). Challenged with the enzyme neprilysin, analogs **K-Aib-1**, **K-Aib-2** and **K-Aib-4** are 17-, 5- and 10-fold more resistant than the standard kinin sequence. The same analogs proved to be 24-, 45- and 9-fold more resistant to hydrolysis by the enzyme angiotensin-converting enzyme (ANCE), respectively. When challenged with the Leucine amino peptidase (also known as aminopeptidase M), the analog **K-Aib-4** was 95-fold less susceptible to hydrolysis.

3.2. Malpighian tubule secretion assay

The *in vitro* diuretic assays measuring *Aedes* Malpighian tubule fluid secretion rates demonstrated that analog **K-Aib-1**, is more potent than the control, FFFSWGa and as active as native *Aedes* kinin 1, NSKYVSKQKFYSWGa at 0.1 nM (Fig. 4).

Table 1
Rate of hydrolysis with three peptidases of the three most potent Aib-containing insect kinin analogs and the control agonist.

Substrate	Enzyme rate of hydrolysis (pmol/h/ng of enzyme)[a]		
	Neprilysin	ANCE	Leu-aminopeptidase[b]
FFFSWGa	12	582	1050
K-Aib-1	0.72	24	33
K-Aib-4	1.2	13	11
K-Aib-2	2.3	65	4.3

[a] Rates are expressed as the mean of at least three assays with a standard error of less that 10%.

[b] LAP Leucine aminopeptidase is also called aminopeptidase M and N and is a general aminopeptidase, but prefers hydrophobic side chains at position 1.

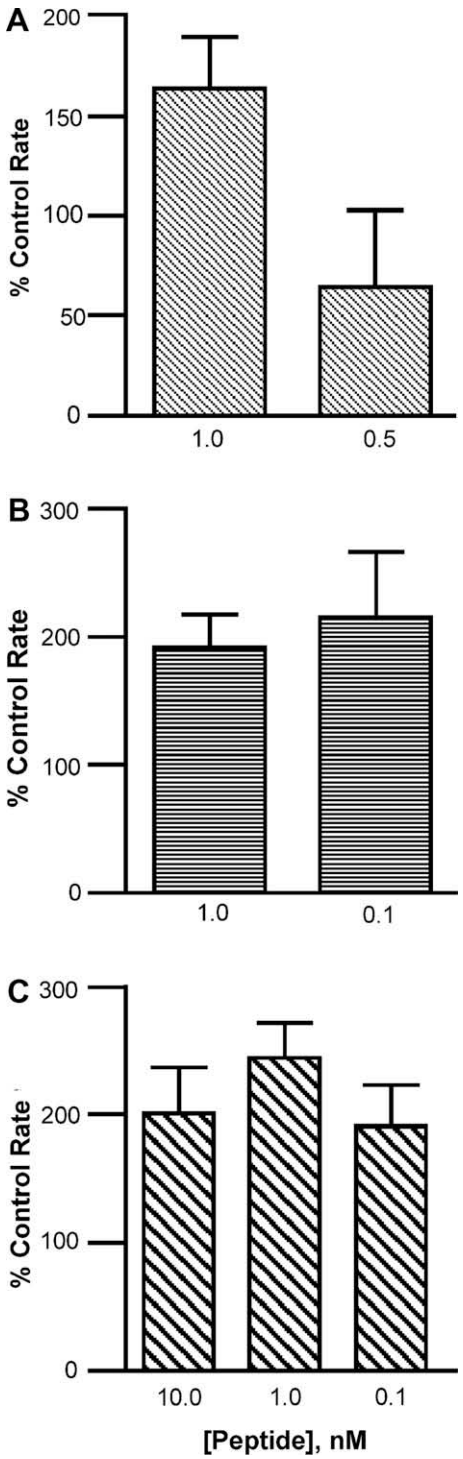

Fig. 4. *In vitro Aedes aegypti* Malpighian tubule secretion assay of control, FFFSWGa (A), native *Aedes* kinin 1 (B) and biostable analog **K-Aib-1** (C). Results are expressed as a percentage of the control rate of secretion measured prior to peptide addition. Bars indicate the mean and vertical lines +1SEM of 4–5 replicates.

4. Discussion

Previous studies demonstrate that in addition to hemolymph aminopeptidases, the insect kinins are hydrolyzed by tissue-bound peptidases at both a primary and secondary site in the peptide chain. The primary hydrolysis-susceptible site lies within the insect kinin C-terminal pentapeptide core region between the Ser^3 (or Pro^3) and conserved Trp^4 residues. A secondary site is found just outside of the core region at the peptide bond N-terminal to Phe^1 (Fig. 1). Furthermore, it has been established that the peptidase angiotensin-converting enzyme (ACE or ANCE) cleaves insect kinin peptides at both the primary and secondary sites. The peptidase neprilysin cleaves the insect kinins at the primary hydrolysis site. Replacement of Ser^3 (or Pro^3) with an unnatural, sterically bulky residue Aib leads to analogs that not only mimic a critical β-turn conformation but also blocks tissue-bound peptidase, ANCE, and neprilysin hydrolysis at the primary site. Previous studies in cell lines expressing the tick and mosquito receptors indicated that the core Aib insect kinin analog FF[Aib]WGa retains significant potency, demonstrating more potency than the control agonist FFFSWGa in tick receptor cell lines and approaching the potency of FFFSWGa in mosquito receptor cell lines (Taneja-Bageshwar et al., 2006). However, while this core Aib analog protects the primary hydrolysis-susceptible site, it cannot protect the secondary site in extended analogs. Notably, the N-terminus of this short analog is vulnerable to hydrolysis by aminopeptidases, which also leads to inactivation (Fig. 1). Furthermore, this analog is significantly less potent than the native *Aedes* kinins, as the mosquito receptor prefers analogs that are extended beyond the C-terminal pentapeptide core. Extended insect kinin analogs would also require additional protection from endopeptidase attack at the secondary site. For these reasons, we prepared a series of biostable Aib-containing analogs that incorporate a second modification (a second Aib residue) to the residue N-terminal to Phe^1 of the core that has been shown to protect the secondary hydrolysis-susceptible site (Fig. 1). For instance, although a non-protected insect kinin was degraded within 1 h by tissue-bound peptidases, the disubstituted Aib kinin analog [Aib]FS[Aib]WGa was found to be completely impervious even up to 4 h, at which time the experiment was terminated (Nachman et al., 2002). In addition, each of the analogs features a sequence that either terminates in a bulky Aib residue or is capped at the N-terminus with an acetyl group to prevent hydrolysis by aminopeptidases. In this study, experiments with ANCE, neprilysin, and Leucine aminopeptidase (aminopeptidase M) indicate that **K-Aib-1**, **K-Aib-2** and **K-Aib-4** demonstrate a highly significant increase in resistance to hydrolysis as compared with the standard kinin FFFSWGa (Table 1). The analog **K-Aib-4** was 95-fold less susceptible to hydrolysis by aminopeptidase. This result was expected, as it is capped with an acetyl group on the N-terminus. Interestingly, uncapped analogs **K-Aib-1** and **K-Aib-2** also demonstrated resistance to hydrolysis by the aminopeptidase, with rates of hydrolysis 32-fold and 244-fold lower, respectively, than the standard insect kinin sequence. The marked resistance to aminopeptidase hydrolysis is likely due to the steric hindrance of the α,α-disubstituted nature of the amino acids Aib and α-MePhe located at the N-terminus.

This series of Aib analogs was tested on tick and mosquito receptor expressing cell lines. An analysis of the biostable Aib analogs showed that all of them elicit a very strong bioluminescence response in both tick and mosquito receptor expressing cell lines. Analog **K-Aib-1**, [Aib]FF[Aib]WGa was found to be the most active, demonstrating a statistically significant higher potency to the control agonist FFFSWGa in both receptor systems. In the mosquito this double Aib analog proved to be much more potent than the single Aib analog and was statistically different from the control, FFFSWGa. Indeed, the EC_{50} of analog **K-Aib-1** indicated that it was eight times more potent than the control agonist and, remarkably, more than 3-fold more active than the natural *Aedes* kinin-II, EC_{50} = 164 nM (Taneja-Bageshwar et al., 2008b), representing a milestone as the first such biostable analog to either match and/or exceed the activity of insect kinins native to mosquitoes or any other arthropod pest. Analog **K-Aib-1** proved to be greatly superior to a previously reported insect kinin analog incorporating

Table 2
Estimated potencies (EC_{50} ± 95% confidence intervals) of Aib analogs that are more or equal in potency to control peptide, FFFSWGa tested on tick (BmLK3) and mosquito (E10) receptor transfected cell lines.

α-Amino isobutyric acid analogs	Tick receptor, (BmLK3 cell line) EC_{50} ± C I (nM)	Mosquito receptor, (E10 cell line) EC_{50} ± C I (nM)
K-Aib-1, [Aib]FF [Aib]WGa	49 ± 12	76 ± 18
Aedes Kin-2[a]		164 ± 30
K-Aib-2, [α MeF]FF [Aib]WGa	111 ± 30	289 ± 50
K-Aib-3, Ac-R[Aib]FF [Aib]WGa	112 ± 40	625 ± 45
K-Aib-4, AcR[β^3 F]FF [Aib]WGa	211 ± 30	411 ± 70
FFFSWGa	271 ± 60	617 ± 50

EC_{50} are an estimate of the concentration required to induce a half-maximal response.
[a] From Taneja-Bageshwar et al., 2008b.

two β-amino acids to protect the primary and secondary proteolytic sites. The β-amino acid analog demonstrated a higher EC_{50} value indicating it was 2-fold less potent than the control agonist in the tick receptor and at least 4-fold less potent than native insect kinins in the mosquito receptor (Taneja-Bageshwar et al., 2008b). Furthermore, this disubstituted β-amino acid analog demonstrated only 65% of the maximal response of insect kinins, in contrast with the 100% maximal response observed for analog **K-Aib-1** in assays with both the arthropod receptor expressing cell lines.

Three other Aib analogs in this study, **K-Aib-2** ([αMeF]F-F[Aib]WGa), **K-Aib-3** (Ac-R[Aib]FF[Aib]WGa), and **K-Aib-4** (Ac-R[β^3F]FF[Aib]WGa) had EC_{50} values that either match or exceed the activity of the control agonist in both receptor assays, but were less potent than the native *Aedes* kinin-II in the mosquito receptor (Table 2). In contrast, analog **K-Aib-6** ([Aib-Aib-Aib-Aib]RF-F[Aib]WGa), containing 4 Aib residues appended to the N-terminus of the insect kinin hexapeptide core, proved to be the least active of this series of six Aib-containing analogs in both receptors. The string of bulky Aib analogs present in the N-terminal region of this analog likely interferes with optimal interaction of the analog with the two arthropod kinin receptors. When tested for Malpighian tubule diuretic activity, analog **K-Aib-1** proved to be as active as native *Aedes* kinin 1 at 0.1 nM and more potent than the control agonist, FFFSWGa.

Several of these potent, biostable analogs, particularly analog **K-Aib-1**, represent ideal new tools for arthropod endocrinologists studying insect kinin-regulated processes, particularly in ticks for which a role for the insect kinins has yet to be established. When challenged with the degradative enzymes ANCE, neprilysin and Leucine aminopeptidase, **K-Aib-1** is 17-, 24- and 32-fold more resistant, respectively, than the control insect kinin sequence (Table 1). While natural insect kinins are subject to rapid inactivation by degradative peptidases in the hemolymph and tissues of arthropods, the potent, biostable analogs presented here would demonstrate longer hemolymph residence times, making them particularly suitable for the study of *in vivo* physiological and behavioral effects of this important class of neuropeptides. Furthermore, these analogs, either in isolation or in combination with biostable analogs of other neuropeptide classes that also regulate aspects of diuretic, antidiuretic, digestive, reproductive and/or developmental processes, represent potential leads in the development of selective, environmentally friendly pest arthropod control agents capable of disrupting those critical processes.

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